Journal of Forestry Research (2012) 23(3): 425-428
DOI 10.1007/s11676-012-0231-y

ORIGINAL PAPER

DNA methylation in tissues of *Chamaedorea elegans*

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Received: 2011-01-07; Accepted: 2011-06-20

Abstract: DNA methylation plays a crucial role in regulating plant development and tissue differentiation. In this study, we compared the methylation levels in leaf, root, and stem in *Chamaedorea elegans* by using the technique of methylation-sensitive amplified fragment length polymorphism AFLP. Over 19% (42/220) bases were uniformly methylated in these tissues. The percentages of polymorphism resulting from varied methylation in mature leaf (L1), young leaf (L2), baby leaf (L3), stem (S), young root (R1) and lignified root (R2) were 29.5%, 29.0%, 27.1%, 30.7%, 63.0% and 28.3%, respectively. The numbers of polymorphic loci detected in the leaves of three developmental stages were similar, ranging from 20 to 30. In contrast, roots at the two developmental stages differed greatly, with 145 polymorphic loci detected in R1 and 27 in R2. Our results suggest that the methylation level in leaves slightly increases with aging, while that in roots decreases dramatically with aging.

Keywords: DNA Methylation; *Chamaedorea elegans*; tissue; root; leaf; stem

Introduction

DNA methylation is an important post-replicative modification that occurs in the residues of cytosine and refers to the addition of a methyl group to five positions of the cytosine pyrimidine ring.. DNA methylation plays vital roles in various biological processes, including maintenance of genome stability, repression of endogenous retrovirus, X chromosome inactivation, genomic imprinting and developmental gene regulation (Bird 2002; Li 2002). The most important biological consequence of this modification event is that it controls gene regulation. Methylation can provide information directing where and when the gene should be expressed without changing the primary nucleotide sequence (Chela et al. 1990; Dahl et al. 2003). In plants and animals, this kind of DNA methylation is species-, tissue- and organelle-specific. It changes with age and is regulated by hormones (Vanyushin 2006). In DNA of higher plants, up to 30% of cytosine residues are methylated (Gruenbaum et al. 1981). The level of DNA methylation is modulated during plant development and this plays an essential role in the progress of organ or tissue differentiation (Hepburn et al. 1987; Vergara et al. 1990) and stem cell differentiation into various progeny (Thyagarajan et al. 2009). DNA methylation in endosperm tissue was analysed by Bùžek et al. (1998) by using immunolabelling with an antibody raised against 5-methylcytosine (anti-5-mC). They demonstrated that the DNA methylation level changed during endosperm development and mitosis cycles.

We adopted the amplified fragment length polymorphism (AFLP) technique for analysis of methylation by using isoschizomers that show differential sensitivity to cytosine methylation. In order to detect DNA methylation sites, two isoschizomer enzymes Acc65I and KpnI were used to digest DNA. They differ in their sensitivity to DNA methylation (Bednarek et al. 2007). Enzymes recognize the 5' GGTACC 3' site and cut unmethylated DNA. Acc65I is sensitive to CpG methylation while KpnI is insensitive to methylation. We combined both enzymes with the methylation-insensitive MseI. We were able to detect methylation differences by employing this isoschizomer combination.

Chamaedorea is the largest palm genus in the Neotropics, comprising 80–100 species (Bridgewater et al. 2006). *Chamaedorea* occurs as an important understory component in rain forest, and the genus has attracted much interest due to its economic and social value. *Chamaedorea elegans* is one of the important genera in Chamaedorea family. In this study, we compared the methylation levels of different tissues of *C. elegans*. Our results illustrate the change of epi-modification of DNA methylation during individual development.

Foundation project: The study was financially supported by the Natural Science Foundation of China (30800879).

The online version is available at http://www.springerlink.com

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Responsible editor: Yu Lei

Material and methods

Material selection

Samples of *C. elegans* were ten years old and cultivated in nutrient solutions in the greenhouse at Zhejiang Agriculture and Forestry University in Lin'an, Zhejiang, China. Six samples, representing three major plant tissues, including leaf, root and stem of *C. elegans*, were collected on 20 May, 2010. Among the samples, L1, L2, and L3 were ten-year, three-year and newly spread leaves, respectively. R1 and R2 were samples from young roots (including root tips and lignified roots) and unlignified roots. S refers to stem.

DNA isolation

The tissues of leaf, root and stem from *C. elegans* were processed for DNA isolation immediately after collection. Total genomic DNA was isolated from 200 mg of fresh leaves, roots, and stems using the CTAB method (Murray et al. 1980). All reagents used in the study were guaranteed grade.

AFLP analysis

We followed the standard AFLP procedure as described by Vos et al. (1995). Genomic DNA was separated into two parts: one was digested by enzymes Acc65I/MseI, and the other by KpnI/MseI, followed by adaptor ligation, pre-selection and selective amplification steps. All primers used were synthesized by Nanjing Jinsite Biological Engineering and Technology Company in China (primer sequences are shown in Table 1). PCR was performed in a 20 μL reaction system containing 50 ng of template DNA, 0.5 μmol· L^{-1} of each primer, 200 mol· L^{-1} of each dNTP, 1.5 m mol· L^{-1} of $MgCl_2$, 1 unit of *Taq* polymerase, and 2 μL of 10×PCR reaction buffer. A touchdown-PCR program (Don et al. 1991) was used: 5 min at 95° C; 10 cycles of 30 s at 95° C, 30 s at 58° C minus 0.3° C per cycle, 1 min at 72° C; 20 cycles of 30 s at 95° C, 30 s at 55° C, 1 min at 72° C; and 7 min at 72° C for a final extension. Each of the reactions was tested twice to confirm the observed bands in each genotype. PCR products were separated on 6% non-denaturing polyacrylamide gel (80 V for 2.5 h). Gels were silver stained following the procedure of Xu et al. (2002).

Data analysis

The AFLP patterns were converted into binomial matrices consisting of either ones or zeros reflecting presence or absence of the respective bands. Only clear and reproducible bands were counted and scored as 1, and the others were scored as 0. A locus was served if there was no less than a "1" at a given DNA ladder marker site, despite of the number of fragments present among six samples within KpnI/MseI and Acc65I/MseI platforms. Polymorphisms refer to presence and absence of fragments at given loci among samples.

Table 1. Adapter and primer sequences

Adapter	Sequence (5'-3')
Adapters *Acc65* I	CTC GTA GCA TGC GTA CA GTA CTG TAC GCA TGC TAC
Adapters *Kpn* I	CTC GTA GCA TGC GTA CAG TAC TGT ACG CAT GCT AC
Adapters *Mse* I	TAC TCA GGA CTC ATA GAC GAT GAG TCC TGA G
Acc65 I /*Kpn* I pre-selective primer	CAT GCG TAC AGT ACC A
Mse I pre-selective primer	GAT GAG TCC TGA GTA AC
Acc65 I / *Kpn* I selective primer	CAT GCG TAC AGT ACC A TGC xxx
Mse I selective primer	GAT GAG TCC TGA GTA ACxx

xxx – any combination of the nucleotides at the primer 3' ends

Results

DNA methylation levels in six samples

In total, we identified 316 AFLP loci, amplified by five primer combinations in Acc65I/MseI and KpnI/MseI platforms (Table 2). Of these 316 loci, 96 loci failed to display polymorphisms. Among the remaining 220 loci, 42 loci displayed polymorphisms at given loci between Acc65I/MseI and KpnI/MseI platforms (Fig. 1). We defined polymorphisms between platforms as cases where fragments were present in all six samples in one platform but absent in all six samples in another platform. Approximately 19% (42/220) polymorphic loci were detected between Acc65I/MseI and KpnI/MseI platforms and these resulted from methylated variation among the six samples,

Table 2. Comparison of polymorphisms among six samples

Samples	Total loci	Polymorphic loci (%)	Specified polymorphic loci*
L1(aging leaf)	241	71/ 29.5%	2
L2 (young leaf)	238	69/ 29.0%	0
L3 (baby leaf)	236	64/ 27.1%	1
S (stem)	238	73/ 30.7%	4
R1 (young roots)	297	187/ 63.0%	124
R2 (lignified roots)	244	69/ 28.3%	8

*Fragment and polymorphism just present in this sample at the same loci.

Comparison of DNA methylation levels among the samples

Polymorphism (including those uniformly methylated loci in the six samples mentioned above) resulted from variation in DNA methylation in DNA samples examined by Acc65I/MseI and KpnI/MseI platforms. The ratio of polymorphisms resulting from methylation variation was around 30% in leaf, stem, and lignified roots (Table 2). However, the values in the young roots were approximately double (63%). In addition, the specified poly-

morphic loci were significantly higher in young roots (124) as compared to the other five samples.

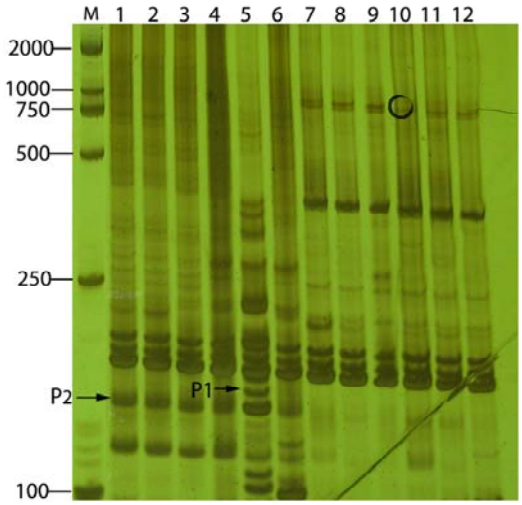

Fig. 1 Products screened with primer AK2-MS1 in *C. elegans* by non-denaturing polyacrylamide gel (M DNA Land Marker DL2000). From 1 to 6 are six samples separated by Acc65Ⅰ/MseⅠ platform; from 7 to 12 are six samples separated by KpnⅠ/MseⅠ platform. Six samples sequence are L1, L2, L3, S, R1 and R2. P1 refers to specific polymorphism locus; P2 refers to polymorphism between Acc65I/MseI and KpnI/MseI platforms.

DNA methylation level in leaves

Polymorphisms resulting from methylation among leaves (except those loci that also display polymorphism in other samples) are compared in Table 3. The total numbers of polymorphic loci did not differ greatly among leaves, values ranging from 20-30, among which a total of 18 was shared by three samples. There were 4 and 2 specified polymorphic loci among L1 and L3 but none in L2. Seven polymorphic loci were shared by L1 and L2, and 2 by L2 and L3. None was shared by L1 and L3. L1, L2, and L3 represent different ages or life periods of leaves. These results suggest that methylation changes with leaf aging.

Table 3. Polymorphisms compared among leaves

	Total polymorphic loci	Specified polymorphic loci	Shared polymorphic loci
L1	29	4	18 and 7
L2	27	0	18, 7 and 2
L3	22	2	18 and 2

DNA methylation levels in roots

Polymorphisms in roots (except those loci that also display polymorphisms in other samples) are compared in Table 4. The total numbers of polymorphic loci differed widely between R1 (145) and R2 (27). There were 138 and 20 specified polymorphic loci in R1 and R2, respectively. Only seven polymorphic loci were shared by R1 and R2. These results suggest that the methylation pattern changed dramatically with root age.

Table 4. Polymorphisms compared among roots

	Total polymorphic loci	Specified polymorphic loci	Shared polymorphic loci
R1	145	138	7
R2	27	20	7

Discussion

In this study, we analyzed the methylation variation in tissues of *C. elegans*. The samples used in this research were from the one *C. elegans* palm grown in controlled conditions. Samples differed only in terms of age and represented tissue development stages. Therefore, our results reflect methylation levels at different developmental ages and different stages of tissue differentiation. DNA methylation changes with age and is regulated by hormones (Vanyushin 2006). In this study, we found that methylation in leaves increases slightly with age. The numbers of polymorphic loci were 22, 27 and 29 in the leaf of newly emerged shoots, 3-year old leaves, and 10-year old leaves, respectively. Seven polymorphic loci were shared by leaves of 10- and 3-year old leaves, and 2 by 3-year old and new leaves. The methylation level appeared to change gradually with age in leaves.

Gruenbaum et al. (1981) reported that in DNA of higher plants, up to 30% of cytosine residues are methylated. The present study showed polymorphism in five of six samples to be at about the same level, including aging leaf (29.5%), young leaf (29.0%), baby leaf (27.1%), stem (30.7%) and lignified roots (28.3%). But a significantly higher level of polymorphism was found in young roots (63.0%). The young roots used in this study (R1) include root tips and non-lignified roots. They represent two developmental stages. According to gene ontology analysis (Fouse et al. 2008), methylated genes and unmethylated genes in stem cells can be divided into two groups. Unmethylated genes are enriched with functions related to transcription, protein and RNA metabolism processes, and some house-keeping and pluripotency roles. The roles of this type of genes are mainly in the maintenance of stem cell properties In contrast, among methylated genes the enriched roles are related to differentiation and signal transduction. Root tips are engaged in vigorous mitosis, whereas, unlignified roots are in rapid growth. Methylated genes in these two tissues are different, and this probably accounts for the higher frequency of polymorphisms in the R1 samples.

Methylation levels appear to change during *C. elegans* growth. The methylation pattern may differ in young roots. Methylation may control the genes related to differentiation and development of *C. elegans*. To find out and clone these genes could be useful in understanding individual differentiation and development.

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